Intermediate monocytes in acute alcoholic hepatitis are functionally activated and induce IL-17 expression in $CD4^+$ T cells

Running title: Intermediate monocytes in acute alcoholic hepatitis

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Key words: alcoholic hepatitis, monocytes, T cells, glucocorticoids, TLR

Word count: 4920 (inc references and figure legends)

Author contributions: AD, EW, EY and DH conducted the experiments. AD, EW and PL performed data analysis. AD, PC and RL conceived the study. AD, EW, LSB and RL drafted the manuscript. MC, PC and RL supervised the study. AD and RL finalized the manuscript.

Grant Support

AD received a grant from the Plymouth Hospitals NHS Trust Charitable Fund. EW, PL, LSB and RL received support from the National Institute for Health Research (NIHR) Biomedical Research Centre based at Moorfields Eye Hospital NHS Foundation Trust and University College London Institute of Ophthalmology. The views expressed are those of the authors and not necessarily those of the National Health Service, the NIHR or the Department of Health.

ABSTRACT

In humans, the three main circulating monocyte subsets are defined by their relative cell surface expression of CD14 and CD16. They are all challenging to study because their characteristics are strongly context specific and this has led to a range of conflicting reports about their function, which is especially so for $CD14^{++}CD16^{+}$ (intermediate) monocytes. *Ex vivo* cultures are also often confounded by the concomitant use of immunosuppressive drugs. We therefore sought to characterize the phenotype and function of intermediate monocytes in the setting of acute inflammation prior to treatment in a cohort of 41 patients with acute alcoholic hepatitis (AH).

Circulating intermediate monocytes were enriched in patients with AH and had an activated phenotype with enhanced expression of CCR2 and CD206 compared to healthy controls. Pro-inflammatory cytokine expression, including IL-1β and IL-23, was also higher than in healthy controls, but both classical ($CD14^{++}CD16^{-}$) and intermediate monocytes in AH were refractory to TLR stimulation. Compared to healthy controls both AH monocyte subsets had greater phagocytic capacity, enhanced ability to drive memory T cell proliferation in co-culture and skewed $CD4^{+}$ T cells to express an increased ratio of IL-17:IFNγ. Furthermore, liver tissue from AH patients demonstrated an enrichment of monocytes including the intermediate subset compared to controls. These data demonstrate that intermediate monocytes are expanded, functionally activated, induce $CD4^{+}$ T cell IL-17 expression and are enriched in the liver of patients with AH.

Words: 231

KEY POINTS

- Circulating intermediate monocytes are enriched in alcoholic hepatitis (AH) patients.
- AH intermediate monocytes are functionally activated.
- AH intermediate monocytes induce $CD4^+$ T cell IL-17 and are enriched in the liver.

INTRODUCTION

Monocytes consist of a heterogeneous group of myeloid cells with varied function and phenotype. Although they are precursors of tissue resident macrophages and dendritic cells they are also effector cells in their own right (1). Accordingly, they are mobilized from the bone marrow in response to acute inflammatory states such as severe sepsis (2) and play an integral role in responding to pathogen and damage-associated molecular patterns (PAMPs and DAMPs) and shaping $CD4^+$ T cell responses (3, 4). In humans, they are classified on the basis of their cell surface expression of CD14 (lipopolysaccharide [LPS] co-receptor) and CD16 (activatory Fc gamma receptor) into classical ($CD14^{++}CD16^-$), intermediate ($CD14^{++}CD16^+$) and non-classical ($CD14^+CD16^{++}$) subsets (5), each of which is thought to have distinct functions. Currently, the conventional view is that the dominant $CD16^-$ classical subset egress from the bone marrow and are recruited to sites of inflammation where they act as phagocytes and give rise to patrolling pro-inflammatory $CD16^{++}$ cells, with the intermediate monocytes representing a transitional group between these two states (6).

Consistent with this it is known that the $CD14^{++}CD16^+$ intermediate subset is enriched in the peripheral blood of patients with autoimmune conditions including rheumatoid arthritis (7). The role of these intermediate monocytes is however poorly understood, and we therefore sought to interrogate their function in the context of acute inflammation. To achieve this we recruited patients with acute alcoholic hepatitis (AH) as, at its onset, it is an exemplar disease for rapid-onset, severe systemic inflammation. Unlike chronic autoimmune diseases, on presentation it is typically not confounded by concomitant use of immunosuppressive medications such as glucocorticoids (8).

Although it is known that immune activation occurs concurrently with an impaired antimicrobial response in AH (9), and tissue resident hepatic macrophages (Kupffer cells) play an important role in orchestrating the immune mediators of hepatocyte damage (10, 11), little is known about the functional role of monocytes. Nonetheless, alcohol may activate them indirectly by modulating intestinal permeability as a consequence of intestinal dysbiosis (10), resulting in an increased exposure of circulating monocytes to PAMPS such as LPS (11). In this study, we therefore hypothesized that circulating monocytes in the peripheral blood of patients presenting with acute AH would be expanded, phenotypically and functionally pro-inflammatory, in particular with regard to their influence on $CD4^+$ T cells.

MATERIALS AND METHODS

Participants

Regulatory approval for this study was obtained from the UK's National Health Service (NHS) Health Research Authority (07/Q2007/05 and 15/LO/1501) and it was conducted according to the International Council for Harmonization Good Clinical Practice Guidance and the Declaration of Helsinki. Written informed consent was obtained from all study participants.

Patients admitted to University Hospitals Bristol NHS Foundation Trust (UHBristol) and University Hospitals Plymouth NHS Trust with a diagnosis of severe AH were prospectively recruited. Severe AH was defined in accordance with recently published trial standards (12) as recent onset jaundice (within previous 3 months) with a serum bilirubin level > 80 µmol/L in a heavy alcohol drinker (more than 60 (males) or 40 (females) g alcohol per day for more than 6 months) with aspartate aminotransferase / alanine aminotransferase > 1.5 and discriminant function > 32 (13). Peripheral blood was drawn *prior* to commencement of any medical therapy. Clinical and biochemical data were recorded, and survival was determined at day 90.

Control blood samples were obtained both from healthy volunteers (HVs) and age and sex-matched patients with alcohol related liver disease (ALD) in the absence of systemic liver inflammation. Patients with ALD were defined as heavy alcohol consumers (> 60 (males) or > 40 (females) g/alcohol per day for more than 6 months) with a diagnosis of cirrhosis (confirmed on imaging or histology) without severe AH (bilirubin < 80 µmol/L and discriminant function < 32) who had an unplanned hospital admission. Patients with ALD

received usual clinical care. The ALD control group was selected as these patients had many similar characteristics to the AH group (demographics, alcohol consumption, underlying liver disease and unplanned hospital admission). The key difference was the absence of the acute inflammatory state of AH. HVs were recruited from local laboratory and hospital workers who provided a self-declaration that they did not have any chronic health problems. Both ALD and HV donors gave a single sample of peripheral blood.

An historic cohort of AH patients recruited at UHBristol between 2007 and 2010, underwent transjugular liver biopsy and tissue surplus to diagnostic requirement was used for this study. The median time of the biopsy after clinical diagnosis of AH was 9 days (range -1 to 143 days). Only samples taken within 10 days of diagnosis were included in this study. In ALD patients who also underwent liver biopsy for clinical reasons (n=4) surplus tissue was used for this study.

Peripheral blood mononuclear cell (PBMC) isolation and magnetic activated cell sorting

PBMCs were isolated from whole blood by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Cardiff, UK). Autologous $CD4^+CD45RO^+$ memory T cells and monocytes were isolated from PBMCs using a memory $CD4^+$ T cell isolation kit (Miltenyi Biotec Ltd, Surrey, UK) and a pan-monocyte negative selection kit (Miltenyi), respectively, according to the manufacturer's instructions. Classical ($CD16^-$) and intermediate ($CD16^+$) monocyte subsets were subsequently separated using CD16 microbeads (Miltenyi).

Cell sorting by flow cytometry

Flow cytometry was performed using the BD Influx flow cytometer (BD Biosciences, Oxford, UK) based on cell surface expression of CD3, CD4, CD45RO (memory $CD4^+$ T cells) and HLA-DR, CD14 and CD16 (monocyte subsets, $CD14^{++}CD16^-$, $CD14^{++}CD16^+$ and $CD14^+CD16^{++}$). Dead cells were excluded using 7-aminoactinomycin D (7AAD; Thermo Fisher Scientific, Loughborough, UK). Monocytes were classified into three subsets according to the recommendations of an expert consensus panel based on CD14 and CD16 expression (5). The gating strategy was performed according to a previously published report, which demonstrated accurate classification of monocytes in patients with acute liver disease (14) and is shown in Supplementary Figure 1A. Purity of sorted cell populations was assessed by flow cytometry and was > 95% (data not shown). Details of antibodies are found in Supplementary Table I.

Cell characterization by flow cytometry

PBMCs were incubated with antibodies to CD3, CD4, CD14, CD16, CCR2, CCR5, CX_3CR1, HLA-DR, CD206 and CD80 then washed and analyzed on a BD LSR II flow cytometer (BD Biosciences). Gating was performed using 'fluorescence minus one' controls for each fluorochrome. Details of antibodies are found in Supplementary Table I. Monocyte subsets were gated as per the sort strategy but without CD45RO (Supplementary Figure 1A).

Monocyte subsets sorted by flow cytometry were cultured at 0.5×10^6 / ml in complete media (RPMI supplemented with 10% fetal bovine serum [GE Healthcare], L-glutamine [Sigma-Aldrich, Poole, UK] and penicillin/streptomycin [Sigma-Aldrich]) in the presence or absence of 100 pg/ml LPS (Sigma-Aldrich) for 18-24h with the addition of 1 µg/ml BD GolgiPlug (BD Biosciences) in a 37°C, 5% CO_2 humidified incubator. Cells were permeabilized and

intracellular staining was performed with antibodies to IL-1β, IL-6, IL-8, IL-12p40, IL-23p19 and TNFα. Gating was performed using 'fluorescence minus one' controls for each fluorochrome. Similar replicates were performed for other TLR ligands: peptidoglycan (TLR2/1) at 1 μg/ml, polyinosinic-polycytidylic (PI:C; TLR3) at 5 μg/ml) and Resiquimod (R848; TLR7/8) at 2.5 μg/ml (all TLR ligands from Invivogen, San Diego, USA). To assess whether endogenous stimuli affected cytokine expression, further replicates of monocyte subsets were stimulated with 200 ng/ml interferon alpha (IFNα; Biolegend, San Diego, USA) using an identical protocol.

Phagocytosis assay

Monocytes isolated by MACS were cultured at 0.5 x 10^6 / ml with 1 ng/ml LPS (Sigma-Aldrich) and 1 x 10^8 fluorescently labelled microparticles (Park Scientific, Northampton, UK) for 10 minutes under tissue culture conditions. Cells were washed and stained with antibodies to CD14 and CD16 before fixation. Phagocytosis of fluorescent microparticles was quantified in each monocyte subset by flow cytometry (Figure 3A) (15).

Monocyte and T cell co-culture assay

Carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher Scientific) labelled T cells and unlabeled monocyte subsets were co-cultured in complete media at a ratio of 5:1 (1x10^5 T cells: 2x10^4 monocytes) for 5 days under tissue culture conditions, in wells pre-coated for 4hr at 37^{0}C with 1 μg/ml anti-CD3 (Thermo Fisher Scientific). A control well without monocytes but containing both anti-CD3 and anti-CD28 (both 1μg/ml; Thermo Fisher Scientific) was also included. For the final 4 hours of culture, cells were pulsed with 20ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), 1μM ionomycin (Sigma-Aldrich)

and 1 µg/ml BD GolgiStop (BD Biosciences). Cells were assessed for intracellular cytokine expression (IL-17A and IFNγ) using flow cytometry (BD LSR II).

Genetic analysis

RNA was prepared from RNA stabilized samples (RNA protect, Qiagen, Manchester, UK) using the RNeasy plus micro kit (Qiagen). RNA was extracted from classical and intermediate monocytes isolated by cell sorting from 3 AH and HV subjects. RNA yields were determined by Nanodrop measurement ($A_{260,}$ Thermo Fisher Scientifc) and RNA integrity was assessed by RNA electrophoresis (Bioanalyser 2100, Agilent, Stockport, UK). Gene expression analysis was carried out using nCounter Human Immunology V2 code set (Nanostring technologies, Seattle, WA, USA). Quality control assessment and normalisation of Nanostring® reporter code counts (RCC) were performed following manufacturer recommended methods using nSolver™ Analysis Software version 4 (Nanostring technologies). Heatmaps were generated for the most differentially expressed genes for each monocyte subset for HV vs AH.

Sample workflow

In all participants, PBMCs were isolated and characterized by CD3, CD4, CD14 and CD16 expression. In a pre-specified analysis, the first 14 HV and AH samples obtained underwent the full surface marker phenotype panel. The subsequent 10 AH patients were analyzed for phagocytosis. Cell sorting experiments required 150 mL fresh blood from each participant. Due to the volume required, we pre-specified an analysis of 10 unselected AH patients.

Liver tissue immunostaining

Pathology specimens were acquired from 7 AH and 4 ALD patients who had undergone transjugular liver biopsy performed by an interventional radiologist at UHBristol. 5 µm sections of the formalin fixed and paraffin embedded tissues samples were obtained using a microtome and mounted onto slides. Samples were deparaffinised in Xylene (Sigma-Aldrich) and rehydrated in ethanol before antigen retrieval in 10 mM citrate buffer and incubating with primary antibodies to CD14 and CD16 (both Abcam, Cambridge, UK) overnight at 4°C. Bound antibodies were detected using fluorescently-labelled secondary antibodies (Abcam), mounted with DAPI (Vector Labs, Peterborough, UK) and fluorescence was visualized under confocal microscopy. Semi-quantitative analysis was performed by manual counting of fluorescent cells per x20 power field in 3 separate portal and lobule regions each. Mean cell counts per field were compared.

Statistical analysis

Continuous variables were compared using Mann-Whitney U tests for independent samples or Wilcoxon signed rank tests for paired samples. Categorical variables were compared using Fisher exact test. All analysis was performed using SPSS version 21 (IBM, New York, USA). Multiparametric cytokine expression was analyzed using SPICE v6.0 (freely available from http://exon.niaid.nih.gov/spice/).

RESULTS

Peripheral blood analyses were conducted on 41 patients with severe AH (mean age 50.2 years, 52% female). Mean disease severity scores of discriminant function (DF) and model for end-stage liver disease (MELD) were 60.8 (standard deviation [sd] 23.9) and 18.1 (sd 6.6) respectively. Overall, there was 14% and 24% 28- and 90-day mortality respectively. Age, gender, disease severity and outcome were similar in 10 patients with ALD who had mean age of 51.8 years, 50% female and MELD 15.1 (sd 5.4). There was 10% and 30% 28- and 90-day mortality respectively (Table 1). Peripheral blood analyses were also performed on 47 HVs.

Intermediate monocytes are enriched in patients with AH

Baseline total monocyte count was significantly greater than the healthy population median (0.71 v 0.60; $p<0.01$; Figure 1A). The proportion of peripheral blood intermediate monocytes, as measured by flow cytometry (Figure 1B) was significantly higher in patients with AH compared to HVs (16.7% v 7.4%; $p<0.001$; Figure 1C). Compared to HVs there is a commensurate reduction in the proportion of classical monocytes (78.4% v 83.0%; $p<0.01$; Figure 1C) and almost an absence in non-classical monocytes in patients with AH compared with HVs (0.7% v 3.9%; $p<0.001$; Figure 1C). The proportions of circulating monocyte subsets in patients with AH were also significantly different to those in patients with ALD (Figure 1C). Classical and intermediate proportions in ALD patients did not differ from HVs (Figure 1C).

Monocyte surface marker phenotype is altered in AH

Flow cytometry was used to quantify surface marker phenotype in AH patients compared to HVs (Figure 1D). Intermediate monocytes from AH patients were phenotypically altered compared to HVs with higher expression of CCR2 (0.6 v 0.3 [expression normalized to HV classical monocytes]; $p<0.01$) and CD206 (3.4 v 1.2; $p<0.01$) and lower expression of HLA-DR (1.8 v 4.9; $p<0.001$). In AH patients, intermediate monocytes expressed lower CCR2 (0.6 v 1.0; $p<0.05$) but higher HLA-DR (1.8 v 0.5; $p<0.001$), CD206 (2.8 v 1.8; $p<0.05$) and CD80 (1.5 v 1.1; $p<0.05$) compared to classical monocytes. Although a small population, non-classical monocytes in AH patients were phenotypically distinct to intermediate monocytes with lower expression of CCR2 (0.2 v 0.8; $p<0.001$) and CCR5 (0.8 v 1.2; $p<0.05$). The percentage of circulating intermediate monocytes was higher and their HLA-DR expression was lower in AH patients who died within 90 days compared to those that survived (23% v 16% and 18,000 v 37,000 mean fluorescence intensity, respectively; both $p<0.05$; Supplementary figure 1B and C).

Monocyte subsets from AH patients are primed to produce pro-inflammatory cytokines but refractory to stimulation

High proportions of unstimulated AH monocytes produce pro-inflammatory cytokines and have greater co-expression of multiple cytokines compared to HV monocytes (Figure 2A). SPICE analysis showed similar cytokine profiles between unstimulated AH classical and intermediate monocytes but increased cytokine expression in HV intermediate versus classical subsets (Figure 2A). Global cytokine expression in AH v HV classical and intermediate subsets was 85% v 41% ($p<0.05$) and 89% v 59% ($p<0.05$) respectively (Figure 2D).

Specific cytokine analysis revealed that classical monocytes in AH patients produce higher levels of IL-6 (37% v 3%; $p<0.05$), IL-8 (64% v 17%; $p<0.05$) and IL-23 (11% v 1%; $p<0.05$) compared with HVs (Figure 2B). Intermediate monocytes from AH patients produce significantly higher levels of IL-1β (72% v 24%; $p<0.05$), IL-8 (64% v 10%; $p<0.05$) and IL-23 (11% v 2%; $p<0.05$) compared with HVs (Figure 2C). HV monocyte subsets have minimal expression of IL-6, IL-12 and IL-23, but do produce low levels of other pro-inflammatory cytokines (Figure 2B and C).

Gene expression analysis comparing unstimulated *ex vivo* AH and HV monocyte subsets revealed a greater number of differentially expressed genes in intermediate than classical monocytes (Supplementary figure 2A-C). Increased expression was noted in a range of chemokines, cytokines and their receptors (Supplementary figure 2B). *CCR2* was the second most differentially expressed gene in AH compared to HV intermediate monocytes after *CLEC4E*, a calcium-dependent lectin involved in innate pattern recognition receptor (Supplementary figure 2B). Consistent with protein analysis, AH intermediate monocytes expressed higher levels of *IL1B* and *IL8* RNA than HVs. Compared to AH classical monocytes, AH intermediate monocytes generally expressed greater levels of genes involved in inflammatory pathways and cell-cell interaction (Supplementary figure 2D-E).

With regard to monocyte capacity to produce pro-inflammatory cytokines, TLR4 stimulation with LPS of classical monocytes increased global cytokine expression by 1.4 fold in HVs compared to no change in AH patients ($p<0.05$; Figure 2E). This was particularly apparent in increased expression of both IL-1β and IL-6 compared to AH classical monocytes (Supplementary Figure 1D). No changes in global cytokine expression were noted in TLR4 stimulated HV or AH intermediate monocytes (Figure 2E). Similar findings were true of

TLR2/1 (peptidoglygan), TLR3 (PI:C) and TLR 7/8 (R848) stimulation (Supplementary Table II). AH classical and intermediate monocytes were also unresponsive to endogenous stimulation with IFNα (Supplementary Table II).

Monocytes from patients with AH are functionally activated

LPS stimulated phagocytosis of fluorescently labelled microparticles was significantly higher in both classical and intermediate monocytes from AH patients compared to HV monocytes (65% v 46% in classical monocytes; $p<0.05$ and 52% v 19%; $p<0.001$ in intermediate monocytes; Figure 3B). In HVs, phagocytic capacity was lower in intermediate compared to classical monocytes (19% v 46%; $p<0.01$; Figure 3B). Of monocytes actively phagocytosing microparticles, intermediate monocytes from AH patients had enhanced ability to phagocytose more than one particle per monocyte compared to HVs (62% v 36%; $p<0.01$; Figure 3C) which was similar to AH classical monocytes (62% v 61%; $p>0.05$).

Classical and intermediate monocytes from AH patients drive similar T cell responses

In patients with AH, classical and intermediate monocytes have similar effects on memory $CD4^+$ T cell proliferation and polarization with equal proliferation (65% v 64%; Figure 4A), IL-17 expression (15.3% v 12.6%; Figure 4B) and IFNγ expression (13.7% v 14.7%; Figure 4C). However, in HVs, intermediate monocytes drove significantly less memory $CD4^+$ T cell proliferation (36% v 46%; $p<0.01$; Figure 4A) and IL-17 expression (6.5% v 8.6%; $p<0.05$; Figure 4B) than classical monocytes and had a higher IFNγ/IL-17 ratio (3.2 v 2.1; $p<0.01$; Figure 4E). Compared to HV intermediate monocytes, those from AH patients drove greater T cell proliferation (64% v 36%; $p<0.05$; Figure 4A) and IL-17 expression (12.6% v 6.5%; $p<0.05$; Figure 4B) with a trend to lower IFNγ expression (4.2% v 18.0%; $p=0.09$; Figure 4C).

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308 **Intrahepatic monocytes**

309 Liver tissue from AH patients, taken a median of 7.5 days after clinical diagnosis was made,
310 demonstrated a significant increase in $CD14^+$ monocytes compared to ALD controls (25.7 v
311 10.5 cells per field; $p<0.01$; Figure 5B). This difference was particularly evident in portal
312 regions in both dual positive $CD14^+CD16^+$ (Figure 5C) and single positive $CD14^+CD16^-$
313 (Figure 5D) cells versus ALD patients (6.3 v 1.3 cells per field; $p<0.05$ and 15.7 v 9.0 cells
314 per field; $p<0.05$ respectively).

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DISCUSSION

In this study, we have demonstrated an enrichment of intermediate monocytes in the peripheral blood of patients with acute severe AH. These are characterized by the upregulation of CCR2 and expression of pro-inflammatory cytokines. They are also refractory to TLR stimulation. Furthermore, intermediate monocytes are functionally similar to classical monocytes in patients with AH. Compared to HVs, both subsets have an activated phenotype with greater phagocytic capacity, enhanced ability to drive memory T cell proliferation in co-culture and favor a Th17 phenotype. Liver tissue from AH patients demonstrates an enrichment of monocytes including the intermediate subset compared to ALD controls. This leads us to hypothesize that activated circulating monocytes home to the liver and contribute to disease pathogenesis.

Severe AH is associated with an enrichment of circulating intermediate monocytes, as described in other inflammatory diseases (7, 8, 14, 16), which is not a result of either alcohol related cirrhosis or active heavy alcohol use itself (Figure 1C). Both the percentage of circulating intermediate monocytes and their activation status (HLA-DR expression) are associated with clinical outcome (Supplementary figure 1B and C). Protein and gene expression and functional analyses have enabled characterization of these monocytes in comparison to HVs. Based on the canonical cell surface markers for classical (CCR2), intermediate (CCR5) and non-classical monocytes (CX_3CR1) (14, 17-19), the elevated expression of CCR2 in AH intermediate monocytes suggest that they have the ability to migrate to the inflamed liver, consistent with our liver immunostaining findings. CCL2, the ligand for CCR2, is highly expressed in AH liver tissue (20). Furthermore, in comparison to HV intermediate monocytes, CCR5 expression is downregulated in AH intermediate

monocytes. Reduced CCR5 and elevated CCR2 expression are indicative of an immature monocyte phenotype (21, 22), suggesting that these monocytes have recently egressed from the bone marrow as part of the inflammatory response. Other surface proteins involved in cell-cell interactions were also upregulated including CD80 and CD206, consistent with their enhanced phagocytosis and proliferative T cell drive (Figures 3 and 4). Furthermore, AH intermediate monocytes express greater levels of genes involved in inflammatory pathways and cell-cell interactions compared to classical monocytes suggesting an activated phenotype (Supplementary figure 2).

AH monocytes are functionally activated. Intermediate monocytes from AH patients demonstrated high phagocytic capacity (in both the proportion able to phagocytose and the number of particles phagocytosed per monocyte; Figure 3). They secreted high levels of pro-inflammatory cytokines but were refractory to further modulation by a range of bacterial, fungal and viral TLR ligands. Hence, they may already be maximally stimulated *in vivo* and cannot further upregulate their intracellular cytokine expression. Importantly, expression of IL-1β, and IL-23, were higher in classical and intermediate monocytes from patients with AH compared to HVs (Figure 2). As these cytokines are essential for the differentiation of Th17 cells from naïve $CD4^+$ T cells (3, 23), this suggests that these monocytes are skewed to polarize T cells to a Th17 phenotype in the context of acute inflammation.

We tested this *in vitro* and demonstrated that classical rather than intermediate monocytes from healthy volunteers induce greater memory T cell proliferation and more IL-17 expression with a higher IFNγ:IL-17 ratio. This replicated previous findings (8). However, in AH patients, in the context of increased expression of IL-1β and IL-23 from their classical and intermediate monocytes, this was reversed and co-cultured memory T cells had a reduced

ratio of IFNγ:IL-17 and favored IL-17 expression (Figure 4). This is consistent with previous reports in patients with alcohol related liver disease (24, 25), but the consequences of this switch are unclear, as a skew to a Th17 phenotype may either be pro-inflammatory and pathogenic (26), or control disease through the recruitment of neutrophils which enhance hepatic regeneration and are associated with an improved prognosis (27, 28).

Additionally, our data demonstrate an enrichment in intrahepatic monocytes in AH patients compared to ALD controls (Figure 5) suggesting the acute inflammatory state in AH and not just heavy alcohol consumption or cirrhosis itself is associated with intrahepatic monocyte trafficking. Given the increased expression of liver homing chemokine receptors and the activated monocyte phenotype of circulating monocytes it is possible that these are the same cells we have documented within the liver, where they may be contributing to disease pathogenesis. Further studies are required to examine the dynamics of monocyte liver infiltration.

We have compared our findings in patients with AH to healthy controls as well as ALD patients. The latter group were selected as appropriate disease controls as they shared many clinical characteristics with AH patients (ongoing alcohol consumption, demographics, underlying liver disease and were hospitalizsed). This has allowed us to conclude that the differences seen in AH patients were not simply due to alcohol consumption or underlying liver disease but related to the inflammatory state of AH.

There remains debate over the functional phenotype of the intermediate monocyte (29) with observations of similarities to both the classical (30) and non-classical (18) subsets. Some studies have reported that intermediate monocytes are the main producers of pro-

inflammatory cytokines (30) while others that they are anti-inflammatory with high expression of IL-10 in response to TLR4 ligand (31). What is clear is that there is significant heterogeneity of monocytes, which has been well defined in the healthy state (18, 19, 32, 33). Recent single-cell RNA sequencing has further categorized $CD16^+$ monocytes into three distinct subsets with some functional overlap with classical and non-classical monocytes (34). Although the expansion of intermediate monocytes has been reported in several inflammatory conditions and proposed as a biomarker of outcome (35), there are few reports of their function in inflammatory states. Consistent with our findings, the best data are from patients with rheumatoid arthritis in which intermediate monocytes induced IL-17 expression from $CD4^+$ T cells (36). However, further functional phenotyping has not been conducted.

Here were present the first report of the functional phenotype of intermediate monocytes in AH. We conclude that, in AH, intermediate monocytes are functionally activated. The intermediate monocyte subset is expanded in the peripheral circulation, tracks to the site of inflammation and has increased phagocytic capacity and ability to drive both T cell proliferation and IL-17 expression than in normal homeostatic conditions. In this acute inflammatory condition, intermediate monocytes may play a role in disease pathogenesis and, therefore, are candidates for further study in the development of disease-specific biomarkers and targeted immune therapies.

ACKNOWLEDGEMENTS

We thank all the study participants for taking part in this study. In addition, we thank Dr Behrang Mozayani for his assistance in obtaining the fixed liver tissue and Mr Steve Blunden for preparing the tissue for immunostaining. The authors wish to acknowledge the assistance of Dr Andrew Herman for cell sorting and the University Of Bristol Faculty Of Biomedical Sciences Flow Cytometry Facility. We also thank Dr Paul Waines, Ms Justyna Lopatecka and Dr Huey Tan for their assistance in cell sorting.

Conflict of interest disclosure

None of the authors have any conflict of interest to declare

REFERENCES

1. Ginhoux, F., and S. Jung. 2014. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nature reviews. Immunology* 14: 392-404.
2. Van Furth, R., M. C. Diesselhoff-den Dulk, and H. Mattie. 1973. Quantitative study on the production and kinetics of mononuclear phagocytes during an acute inflammatory reaction. *The Journal of experimental medicine* 138: 1314-1330.
3. Acosta-Rodriguez, E. V., G. Napolitani, A. Lanzavecchia, and F. Sallusto. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nature immunology* 8: 942-949.
4. Evans, H. G., N. J. Gullick, S. Kelly, C. Pitzalis, G. M. Lord, B. W. Kirkham, and L. S. Taams. 2009. In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. *Proceedings of the National Academy of Sciences of the United States of America* 106: 6232-6237.
5. Ziegler-Heitbrock, L., P. Ancuta, S. Crowe, M. Dalod, V. Grau, D. N. Hart, P. J. Leenen, Y. J. Liu, G. MacPherson, G. J. Randolph, J. Scherberich, J. Schmitz, K. Shortman, S. Sozzani, H. Strobl, M. Zembala, J. M. Austyn, and M. B. Lutz. 2010. Nomenclature of monocytes and dendritic cells in blood. *Blood* 116: e74-80.
6. Stansfield, B. K., and D. A. Ingram. 2015. Clinical significance of monocyte heterogeneity. *Clin Transl Med* 4: 5.
7. Hepburn, A. L., J. C. Mason, and K. A. Davies. 2004. Expression of Fcgamma and complement receptors on peripheral blood monocytes in systemic lupus erythematosus and rheumatoid arthritis. *Rheumatology* 43: 547-554.
8. Liu, B., A. Dhanda, S. Hirani, E. L. Williams, H. N. Sen, F. Martinez Estrada, D. Ling, I. Thompson, M. Casady, Z. Li, H. Si, W. Tucker, L. Wei, S. Jawad, A. Sura, J. Dailey, S. Hannes, P. Chen, J. L. Chien, S. Gordon, R. W. Lee, and R. B. Nussenblatt. 2015. CD14++CD16+ Monocytes Are Enriched by Glucocorticoid Treatment and Are Functionally Attenuated in Driving Effector T Cell Responses. *Journal of immunology* 194: 5150-5160.
9. Dhanda, A. D., and P. L. Collins. 2015. Immune dysfunction in acute alcoholic hepatitis. *World journal of gastroenterology : WJG* 21: 11904-11913.
10. Betrapally, N. S., P. M. Gillevet, and J. S. Bajaj. 2016. Changes in the Intestinal Microbiome and Alcoholic and Nonalcoholic Liver Diseases: Causes or Effects? *Gastroenterology* 150: 1745-1755 e1743.
11. Thurman, R. G., B. U. Bradford, Y. Iimuro, M. V. Frankenberg, K. T. Knecht, H. D. Connor, Y. Adachi, C. Wall, G. E. Arteel, J. A. Raleigh, D. T. Forman, and R. P. Mason. 1999. Mechanisms of alcohol-induced hepatotoxicity: studies in rats. *Frontiers in bioscience : a journal and virtual library* 4: e42-46.
12. Crabb, D. W., R. Bataller, N. P. Chalasani, P. S. Kamath, M. Lucey, P. Mathurin, C. McClain, A. McCullough, M. C. Mitchell, T. R. Morgan, L. Nagy, S. Radaeva, A. Sanyal, V. Shah, G. Szabo, and N. A. H. Consortia. 2016. Standard Definitions and Common Data Elements for Clinical Trials in Patients With Alcoholic Hepatitis: Recommendation From the NIAAA Alcoholic Hepatitis Consortia. *Gastroenterology* 150: 785-790.
13. Maddrey, W. C., J. K. Boitnott, M. S. Bedine, F. L. Weber, Jr., E. Mezey, and R. I. White, Jr. 1978. Corticosteroid therapy of alcoholic hepatitis. *Gastroenterology* 75: 193-199.
14. Abeles, R. D., M. J. McPhail, D. Sowter, C. G. Antoniades, N. Vergis, G. K. Vijay, E. Xystrakis, W. Khamri, D. L. Shawcross, Y. Ma, J. A. Wendon, and D. Vergani. 2012. CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14(hi) /CD16(neg) monocytes: Expansion of CD14(hi) /CD16(pos) and contraction of CD14(lo) /CD16(pos) monocytes in acute liver failure.

Cytometry. Part A : the journal of the International Society for Analytical Cytology 81: 823-834.

15. Steinkamp, J. A., J. S. Wilson, G. C. Saunders, and C. C. Stewart. 1982. Phagocytosis: flow cytometric quantitation with fluorescent microspheres. *Science* 215: 64-66.
16. Fingerle, G., A. Pforte, B. Passlick, M. Blumenstein, M. Strobel, and H. W. Ziegler-Heitbrock. 1993. The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. *Blood* 82: 3170-3176.
17. Zimmermann, H. W., C. Trautwein, and F. Tacke. 2012. Functional role of monocytes and macrophages for the inflammatory response in acute liver injury. *Frontiers in physiology* 3: 56.
18. Wong, K. L., J. J. Tai, W. C. Wong, H. Han, X. Sem, W. H. Yeap, P. Kourilsky, and S. C. Wong. 2011. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood* 118: e16-31.
19. Zawada, A. M., K. S. Rogacev, B. Rotter, P. Winter, R. R. Marell, D. Fliser, and G. H. Heine. 2011. SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. *Blood* 118: e50-61.
20. Affo, S., M. Dominguez, J. J. Lozano, P. Sancho-Bru, D. Rodrigo-Torres, O. Morales-Ibanez, M. Moreno, C. Millan, A. Loaeza-del-Castillo, J. Altamirano, J. C. Garcia-Pagan, V. Arroyo, P. Gines, J. Caballeria, R. F. Schwabe, and R. Bataller. 2013. Transcriptome analysis identifies TNF superfamily receptors as potential therapeutic targets in alcoholic hepatitis. *Gut* 62: 452-460.
21. Phillips, R. J., M. Lutz, and B. Premack. 2005. Differential signaling mechanisms regulate expression of CC chemokine receptor-2 during monocyte maturation. *Journal of inflammation* 2: 14.
22. Tuttle, D. L., J. K. Harrison, C. Anders, J. W. Sleasman, and M. M. Goodenow. 1998. Expression of CCR5 increases during monocyte differentiation and directly mediates macrophage susceptibility to infection by human immunodeficiency virus type 1. *Journal of virology* 72: 4962-4969.
23. Volpe, E., N. Servant, R. Zollinger, S. I. Bogiatzi, P. Hupe, E. Barillot, and V. Soumelis. 2008. A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nature immunology* 9: 650-657.
24. Gao, B., and A. Waisman. 2012. Th17 cells regulate liver fibrosis by targeting multiple cell types: many birds with one stone. *Gastroenterology* 143: 536-539.
25. Lemmers, A., C. Moreno, T. Gustot, R. Marechal, D. Degre, P. Demetter, P. de Nadai, A. Geerts, E. Quertinmont, V. Vercruysse, O. Le Moine, and J. Deviere. 2009. The interleukin-17 pathway is involved in human alcoholic liver disease. *Hepatology* 49: 646-657.
26. Lafdil, F., A. M. Miller, S. H. Ki, and B. Gao. 2010. Th17 cells and their associated cytokines in liver diseases. *Cellular & molecular immunology* 7: 250-254.
27. Altamirano, J., R. Miquel, A. Katoonizadeh, J. G. Abraldes, A. Duarte-Rojo, A. Louvet, S. Augustin, R. P. Mookerjee, J. Michelena, T. C. Smyrk, D. Buob, E. Leteurtre, D. Rincon, P. Ruiz, J. C. Garcia-Pagan, C. Guerrero-Marquez, P. D. Jones, A. S. t. Barritt, V. Arroyo, M. Bruguera, R. Banares, P. Gines, J. Caballeria, T. Roskams, F. Nevens, R. Jalan, P. Mathurin, V. H. Shah, and R. Bataller. 2014. A Histologic Scoring System for Prognosis of Patients With Alcoholic Hepatitis. *Gastroenterology*.
28. Taieb, J., C. Delarche, V. Paradis, P. Mathurin, A. Grenier, B. Crestani, M. Dehoux, D. Thabut, M. A. Gougerot-Pocidalo, T. Poynard, and S. Chollet-Martin. 2002. Polymorphonuclear neutrophils are a source of hepatocyte growth factor in patients with severe alcoholic hepatitis. *Journal of hepatology* 36: 342-348.
29. Ziegler-Heitbrock, L. 2015. Blood Monocytes and Their Subsets: Established Features and Open Questions. *Frontiers in immunology* 6: 423.

30. Cros, J., N. Cagnard, K. Woollard, N. Patey, S. Y. Zhang, B. Senechal, A. Puel, S. K. Biswas, D. Moshous, C. Picard, J. P. Jais, D. D'Cruz, J. L. Casanova, C. Trouillet, and F. Geissmann. 2010. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* 33: 375-386.
31. Skrzeczynska-Moncznik, J., M. Bzowska, S. Loseke, E. Grage-Griebenow, M. Zembala, and J. Pryjma. 2008. Peripheral blood CD14high CD16+ monocytes are main producers of IL-10. *Scandinavian journal of immunology* 67: 152-159.
32. Boyette, L. B., C. Macedo, K. Hadi, B. D. Elinoff, J. T. Walters, B. Ramaswami, G. Chalasani, J. M. Taboas, F. G. Lakkis, and D. M. Metes. 2017. Phenotype, function, and differentiation potential of human monocyte subsets. *PloS one* 12: e0176460.
33. Thomas, G. D., A. A. J. Hamers, C. Nakao, P. Marcovecchio, A. M. Taylor, C. McSkimming, A. T. Nguyen, C. A. McNamara, and C. C. Hedrick. 2017. Human Blood Monocyte Subsets: A New Gating Strategy Defined Using Cell Surface Markers Identified by Mass Cytometry. *Arteriosclerosis, thrombosis, and vascular biology* 37: 1548-1558.
34. Villani, A. C., R. Satija, G. Reynolds, S. Sarkizova, K. Shekhar, J. Fletcher, M. Griesbeck, A. Butler, S. Zheng, S. Lazo, L. Jardine, D. Dixon, E. Stephenson, E. Nilsson, I. Grundberg, D. McDonald, A. Filby, W. Li, P. L. De Jager, O. Rozenblatt-Rosen, A. A. Lane, M. Haniffa, A. Regev, and N. Hacohen. 2017. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science* 356.
35. Yang, J., L. Zhang, C. Yu, X. F. Yang, and H. Wang. 2014. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark Res* 2: 1.
36. Rossol, M., S. Kraus, M. Pierer, C. Baerwald, and U. Wagner. 2012. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis and rheumatism* 64: 671-677.

FIGURE LEGENDS

Figure 1. Intermediate monocytes are enriched in patients with AH and have an altered phenotype compared to HVs. (A) Absolute monocyte counts at baseline of treatment with glucocorticoids in 41 patients with AH (mean and SD). Normal range for the local population is shown (dashed lines). (B) Representative FACS dotplot of CD14 and CD16 expression on monocytes in a HV and a patient with AH. (C) Monocyte subsets as a percentage of total monocyte numbers in HVs (n=47), patients with AH at initial presentation (n=41) and ALD patients (n=10) for classical, intermediate and non-classical subsets. Scatter dot plots show mean and SEM. (D) CCR2, CCR5, CX_3CR1, HLA-DR, CD206 and CD80 surface protein expression in monocyte subsets in HVs (n=14) and patients with AH (n=14) was quantified by flow cytometry. Mean fluorescence intensity was normalized to HV classical monocyte expression of each marker and expressed as a fold difference. Bars charts show mean with SEM. *p<0.05; **p<0.01; ***p<0.001.

Figure 2. Monocytes from patients with AH express higher levels of pro-inflammatory cytokines than healthy volunteers. Classical and intermediate monocyte subsets from HVs (n=3) and patients with AH (n=5) were sorted by flow cytometry and cultured for 18-24h with or without LPS stimulation before intracellular cytokine expression was measured by flow cytometry. (A) Simplified Presentation of Incredibly Complex Evaluations (SPICE) analysis of intracellular cytokines demonstrates greater cytokine expression and co-expression of multiple cytokines in both classical and intermediate monocytes from AH patients versus HVs. (B) and (C) Unstimulated individual cytokine expression in classical (B) and intermediate (C) monocytes in HVs and patients with AH. (D) Global cytokine expression (total percentage positive for any cytokine) in classical and intermediate

monocytes in HVs and patients with AH. Unstimulated classical and intermediate monocytes secrete more cytokines from AH patients than HVs (85% v 41%; $p<0.05$ and 89% v 59%; $p<0.05$ respectively). (E) Fold change in global cytokine expression in the presence of LPS. Addition of LPS does not yield any difference in cytokine expression AH monocyte subsets or in HV intermediate monocytes. Bars charts show mean with SEM.

Figure 3. AH monocytes have enhanced phagocytic capacity compared to HV monocytes. Monocytes isolated by magnetic bead separation from HVs (n=5) and patients with AH (n=10) were cultured with fluorescent coated microspheres in the presence of LPS. (A) Example flow cytometry dotplot of monocyte subsets cultured with fluorescent microparticles. Histograms demonstrate monocytes positive and negative for fluorescently labelled microparticles from classical and intermediate monocyte subsets. Intensity of fluorescence represents the number of microparticles phagocytosed by each monocyte. (B) The proportion of monocytes with intracellular microparticles was quantified in each monocyte subset by flow cytometry. Monocytes from AH patients were more efficient at phagocytosis compared to those from HVs. (C) The proportion of monocytes from each subset which had phagocytosed 2 or more microparticles each was quantified in monocytes from HVs and patients with AH. Intermediate monocytes from AH patients had greater capacity to phagocytose more than 1 microparticle each compared to those from HVs. Scatter dot plots show mean and SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Figure 4. Monocytes from AH patients drive greater T cell proliferation and IL-17A expression than HV monocytes. Memory T cells and monocyte subsets were isolated from HVs (n=16) and patients with AH (n=5) and co-cultured. T cells were labelled with CFSE and proliferation was determined by CFSE dilution and intracellular cytokine expression

599 determined by flow cytometry. (A) Percentage of $CFSE^{lo}$ memory T cells. (B) and (C).
600 Percentage of single positive IL-17 (B) and IFNγ (C) expressing cells. (D) Percentage of cells
601 co-expressing IL-17 and IFNγ. (E) Ratio of IFNγ/IL-17. Bars charts show mean with SEM.
602 *p<0.05; **p<0.01
603
604 **Figure 5. Intrahepatic monocytes are enriched in patients with AH.** (A) Representative
605 confocal immunostaining images from the portal tract of a liver biopsy from a patient with
606 AH (left) and ALD control (right) with multiple dual staining cells (yellow) seen in the AH
607 sample only. ALD controls are actively drinking patients with cirrhosis but no acute
608 inflammatory component. (B) The mean number of $CD14^+$ monocytes in liver tissue from
609 patients with AH (n=7) and patients with ALD (n=4) taken a median of 7.5 days after
610 commencement of glucocorticoid treatment averaged from 3 portal and 3 lobular fields. (C)
611 There are more $CD14^+CD16^+$ monocytes in portal areas in AH compared to ALD patients
612 (6.3 v 1.3; p<0.05). (D) There are more $CD14^+CD16^-$ monocytes in portal regions in AH
613 compared to ALD patients (15.7 v 9.0; p<0.05). Scatter dot plots show mean with SEM.
614 *p<0.05.

TABLES

Table 1. Participant characteristics for AH and ALD patients at baseline and mortality at days 28 and 90.

	Disease category	
	AH (n=41)	**ALD (n=10)**
Age	50.2 (13.4)	51.8 (9.8)
Gender (%female)	52	50
Bilirubin (µmol/L)	288 (166)	102 (73)
Albumin (g/L)	25.7 (7.1)	30.1 (5.1)
INR	1.6 (0.5)	1.6 (0.5)
Creatinine (µmol/L)	68.4 (40.1)	70.1 (20.0)
Sodium (µmol/L)	133 (53)	128.3 (9.7)
DF	60.8 (23.9)	28.1 (28.4)
MELD	18.1 (6.6)	15.1 (5.4)
28 day mortality (%)	14	10
90 day mortality (%)	24	30

Figure 1

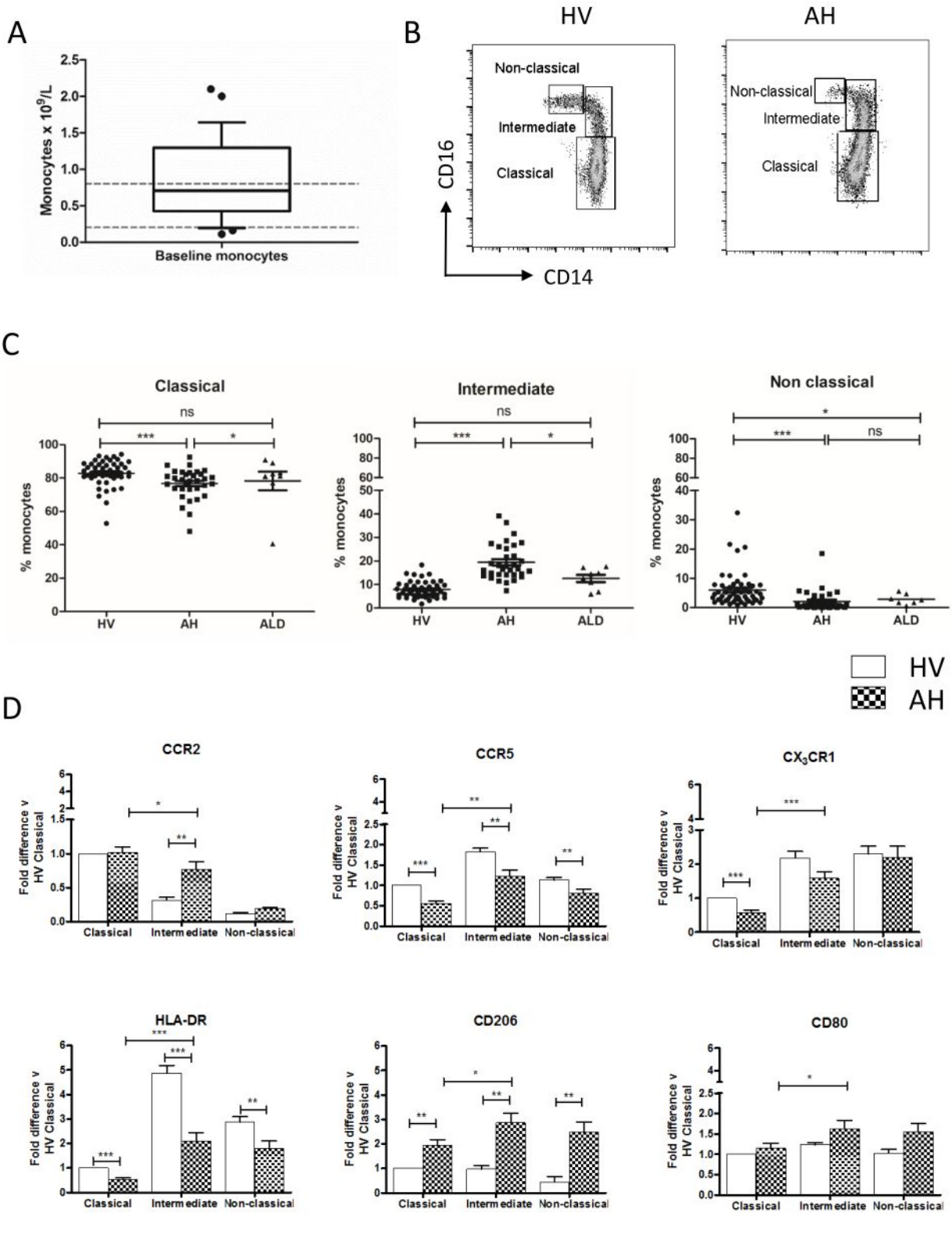

A
Monocytes x 10^9/L
2.5
2.0
1.5
1.0
0.5
0.0
Baseline monocytes
B
HV
AH
Non-classical
Intermediate
Classical
CD16
CD14
C
Classical
Intermediate
Non classical
ns
***
*
% monocytes
HV
AH
ALD
HV
AH
D
CCR2
CCR5
CX3CR1
HLA-DR
CD206
CD80
Fold difference v HV Classical
Classical
Intermediate
Non-classical

Figure 2

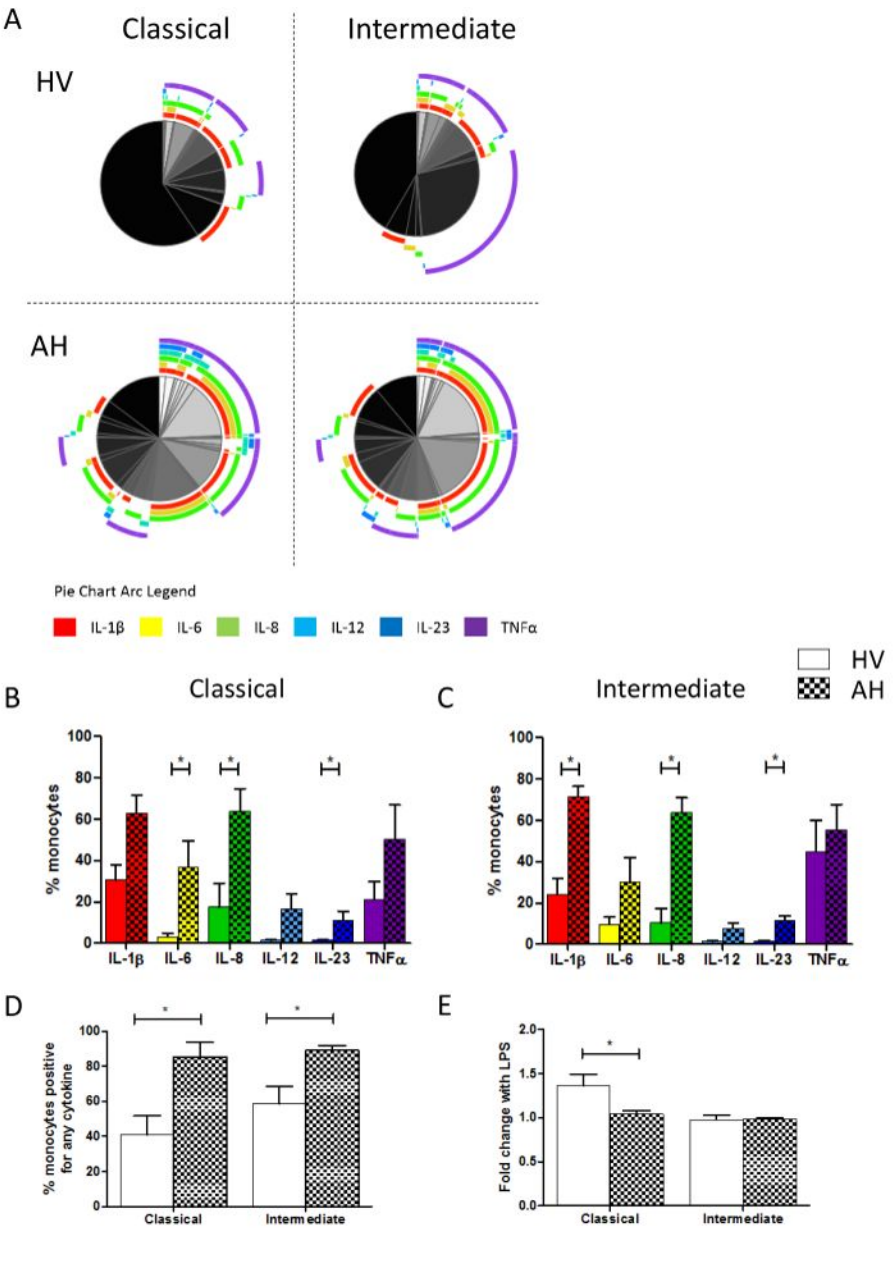

A
Classical
Intermediate
HV
AH
Pie Chart Arc Legend
IL-1β
IL-6
IL-8
IL-12
IL-23
TNFα
B
Classical
C
Intermediate
HV
AH
% monocytes
IL-1β
IL-6
IL-8
IL-12
IL-23
TNFα
D
% monocytes positive for any cytokine
Classical
Intermediate
E
Fold change with LPS
Classical
Intermediate

Figure 3

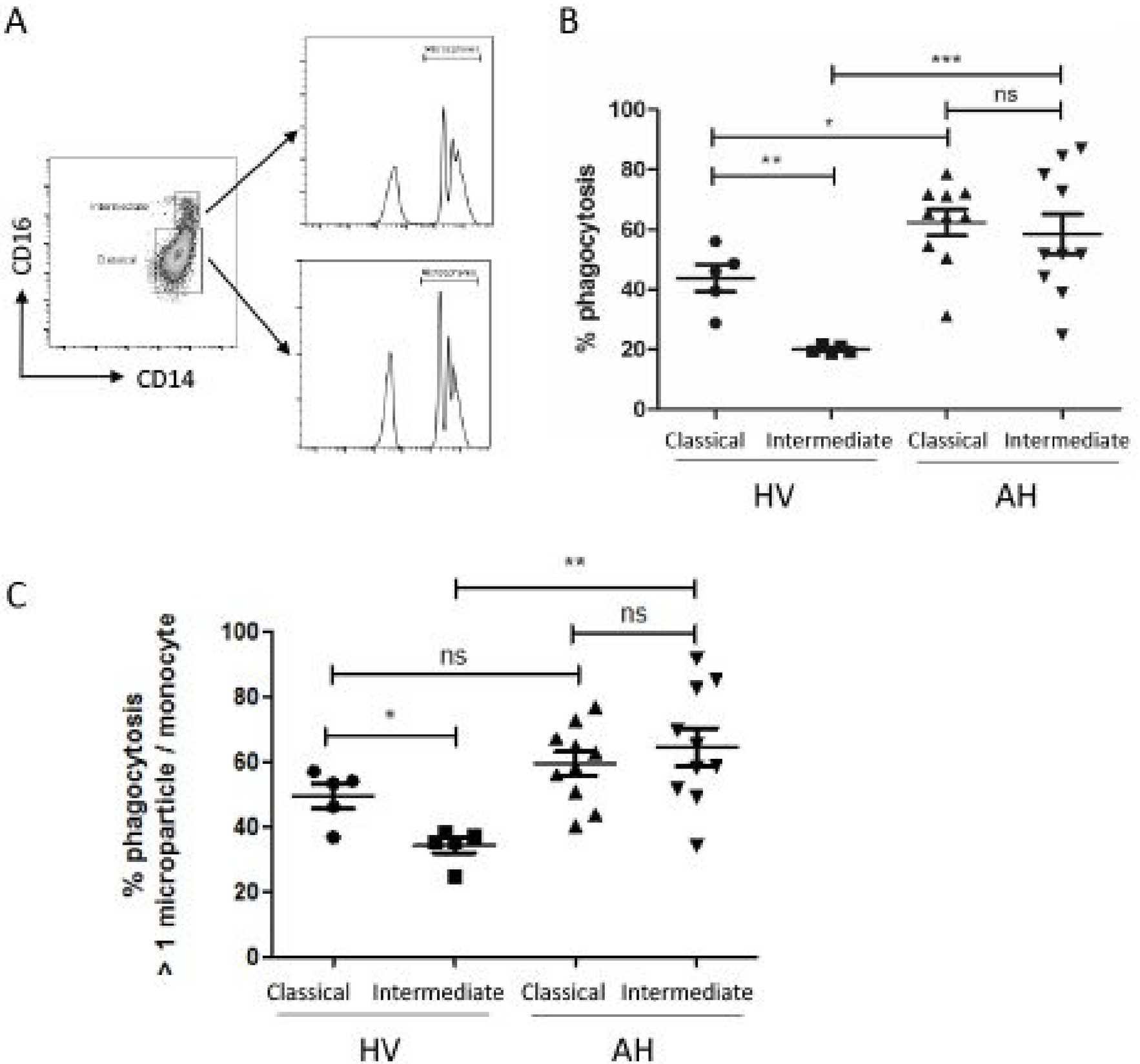

A
CD16
CD14
B
% phagocytosis
100
80
60
40
20
0
**
*
***
ns
Classical
Intermediate
Classical
Intermediate
HV
AH
C
% phagocytosis
> 1 microparticle / monocyte
100
80
60
40
20
0
**
ns
ns
*
Classical
Intermediate
Classical
Intermediate
HV
AH

Figure 4

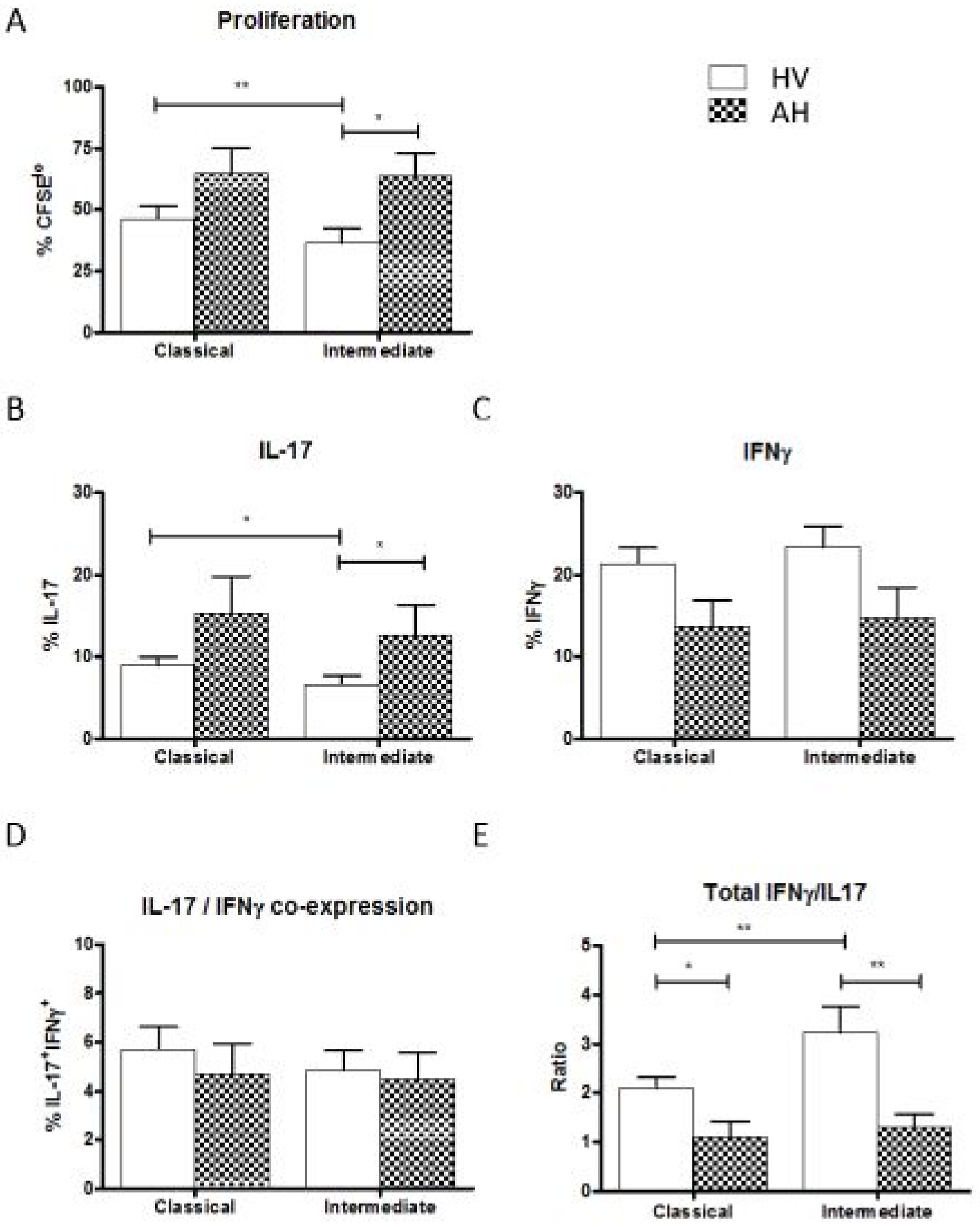
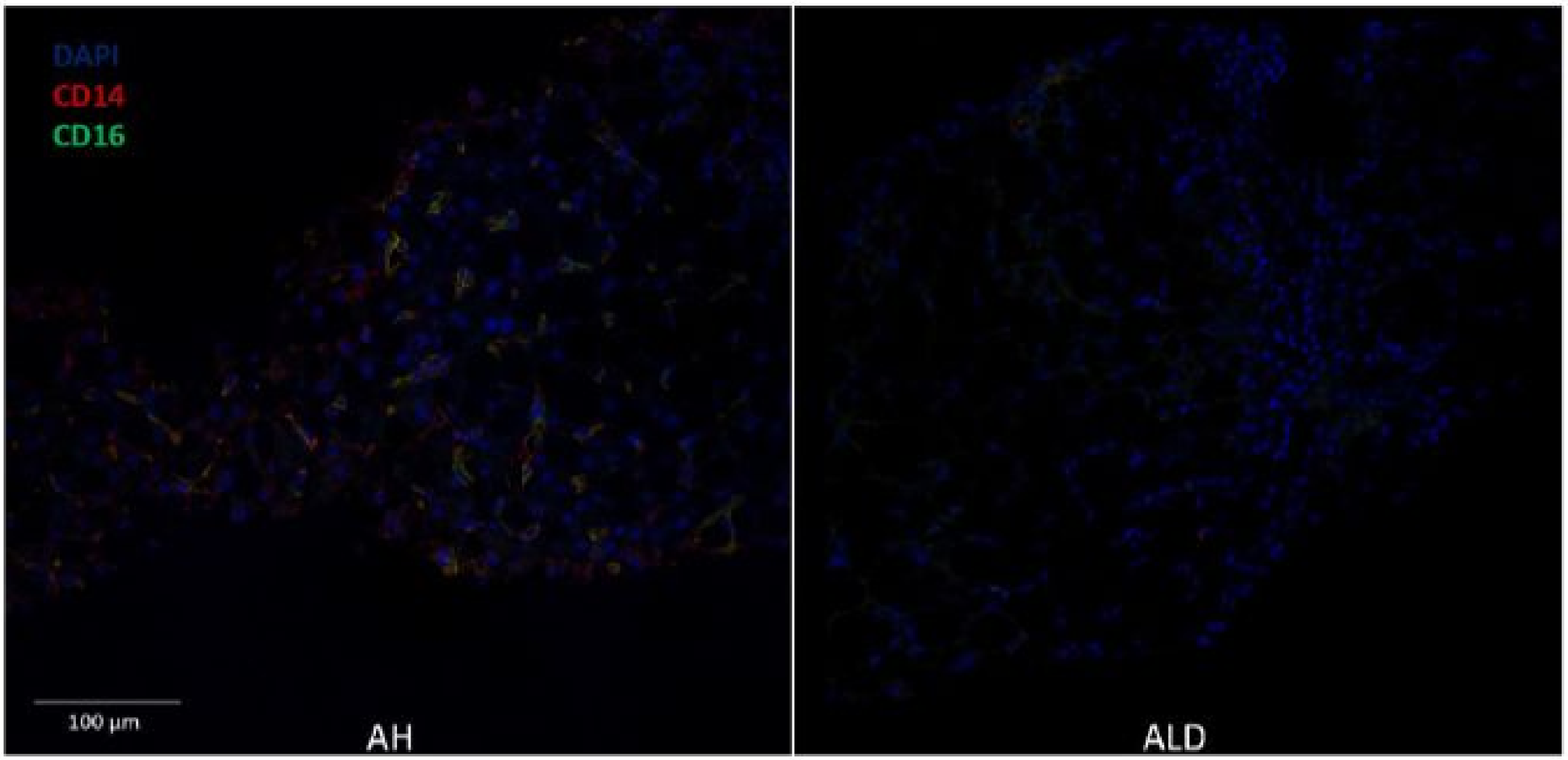
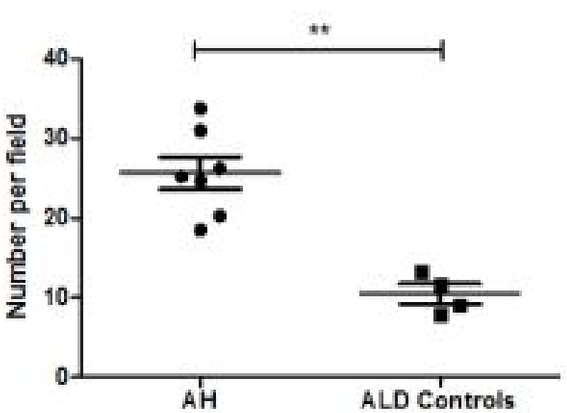

A
Proliferation
HV
AH
% CFSE°
100
75
50
25
0
**
*
Classical
Intermediate
B
IL-17
% IL-17
30
20
10
0
*
*
Classical
Intermediate
C
IFNγ
% IFNγ
30
20
10
0
Classical
Intermediate
D
IL-17 / IFNγ co-expression
% IL-17+IFNγ+
10
8
6
4
2
0
Classical
Intermediate
E
Total IFNγ/IL17
Ratio
5
4
3
2
1
0
**
*
**
Classical
Intermediate

Figure 5

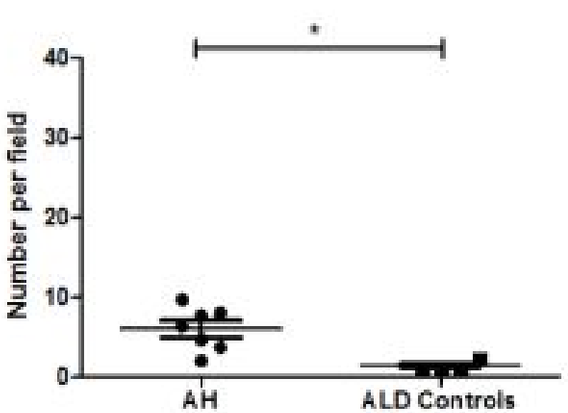
A



B



C



D